# A 45-color panel for comprehensive immunophenotyping of human leukocytes with the Agilent NovoCyte Opteon spectral flow cytometer

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CYTO 2024
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## Introduction

- Agilent has developed the NovoCyte Opteon spectral flow cytometer, which consists of up to five lasers (349nm, 405nm, 488nm, 561nm, and 637nm) and 73 detectors ( 70 fluorescence detection channels, FSC, BSSC, and VSSC).
- Taking an OMIP-069[1] panel as a backbone, a 45-color immunophenotyping panel was designed and optimized for the Agilent NovoCyte Opteon spectral flow cytometer.
- Five additional biomarkers were added for broader research applications **(Table 1)**. These biomarkers are CD31, CD45RO, CD69, CD33 and LAG-3. The addition of CD31 and CD45RO enables the identification of recent thymic emigrants (RTEs) within the CD4+ T cells. CD69 is an early activation marker that is upregulated on T cells upon stimulation. LAG-3 enables the analysis of exhausted T cells together with PD-1. CD33 allows the phenotyping of DCs and monocytes.
- Fluorochromes for four markers in OMIP-069 were replaced **(Table 1)** for optimized results.

## Experimental

### Information of sample preparation and data analysis

Samples were prepared by sequential staining using PBMCs. Data were analyzed using NovoExpress (Opteon) software (v2.0.0) for 2D plots, and FlowJo software (v10.10.0) for high dimensional analysis (UMAP v4.0.4, FlowSOM v4.0.0).

| Marker | Fluorochromes | Marker | Fluorochromes | Marker | Fluorochromes |
|---|---|---|---|---|---|
| CD45RA | BUV395 | CD141 | BB515 | CD159a | APC |
| **CD45RO** | **SBUV445** | CD57 | FITC | CD1c | AF647 |
| CD16 | BUV496 | CD14 | Spark Blue 550 | CD19 | SNIR685 |
| CCR5 | BUV563 | **CD33** | **SBB580** | **CD127** | **SR718** |
| CD314 | BUV615 | **CD223** | **NFB660/120s** | **Viability** | **Zombie NIR** |
| CD39 | BUV661 | CD45 | PerCP | CD27 | APC-H7 |
| CD56 | BUV737 | CD2 | PerCP-Cy5.5 | CD38 | APC-Fire 810 |
| CD8 | BUV805 | TCRγδ | PerCP-eF710 | | |
| CCR7 | BV421 | **CD69** | **SBB765** | **Text in red type:** | |
| CD123 | SB436 | **CD31** | **SBB810** | five added biomarkers | |
| CD11c | eF450 | CD159c | PE | **Text in blue type:** | |
| IgD | BV480 | **CD20** | **Spark YG 593** | modified fluorochromes | |
| CD3 | BV510 | CD337 | PE-Dazzle 594 | **Abbreviation:** | |
| IgM | BV570 | **CD4** | **CF594** | SBUV=StarBright UltraViolet<br>SB=Super Bright<br>SBB=StarBright Blue<br>NFB=NovaFluor Blue<br>eF=eFluor<br>AF=Alexa Fluor<br>SNIR=Spark NIR<br>SR=Spark Red | |
| IgG | BV605 | CD24 | PE-AF610 | | |
| CD28 | BV650 | CD95 | PE-Cy5 | | |
| CCR6 | BV711 | CD25 | PE-AF700 | | |
| CXCR5 | BV750 | CXCR3 | PE-Cy7 | | |
| PD-1 | BV785 | HLA-DR | PE-Fire 810 | | |

Table1. Antibodies used for 45-color panel.

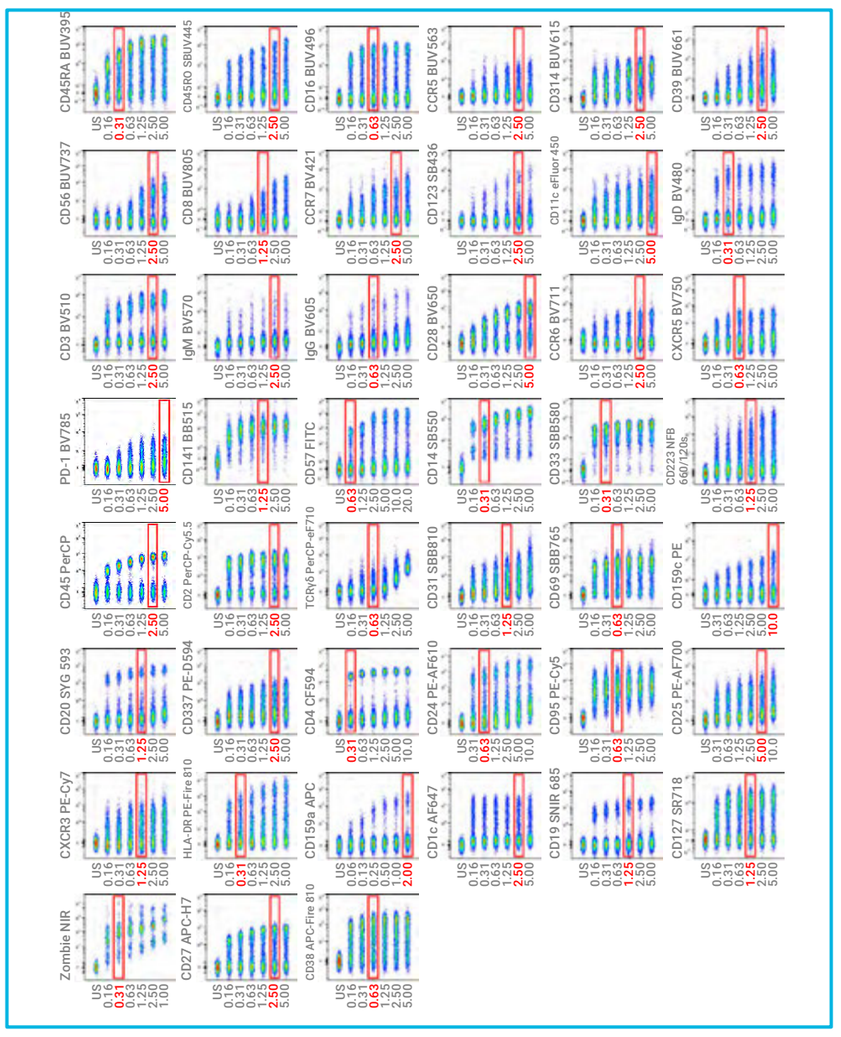


**Figure 1. Antibody titrations for the 45-color panel.** All antibodies were titrated over six concentrations with a starting concentration ranging from 1 to 20 µL per 100 µL staining volume. The optimal titer was chosen based on the Stain Index values (i.e. choosing a higher Stain Index, while considering a lower titer to minimize the spreading error) and is indicated by the red box in the plot.

## Experimental

### Spectral signatures and Similarity Index Matrix

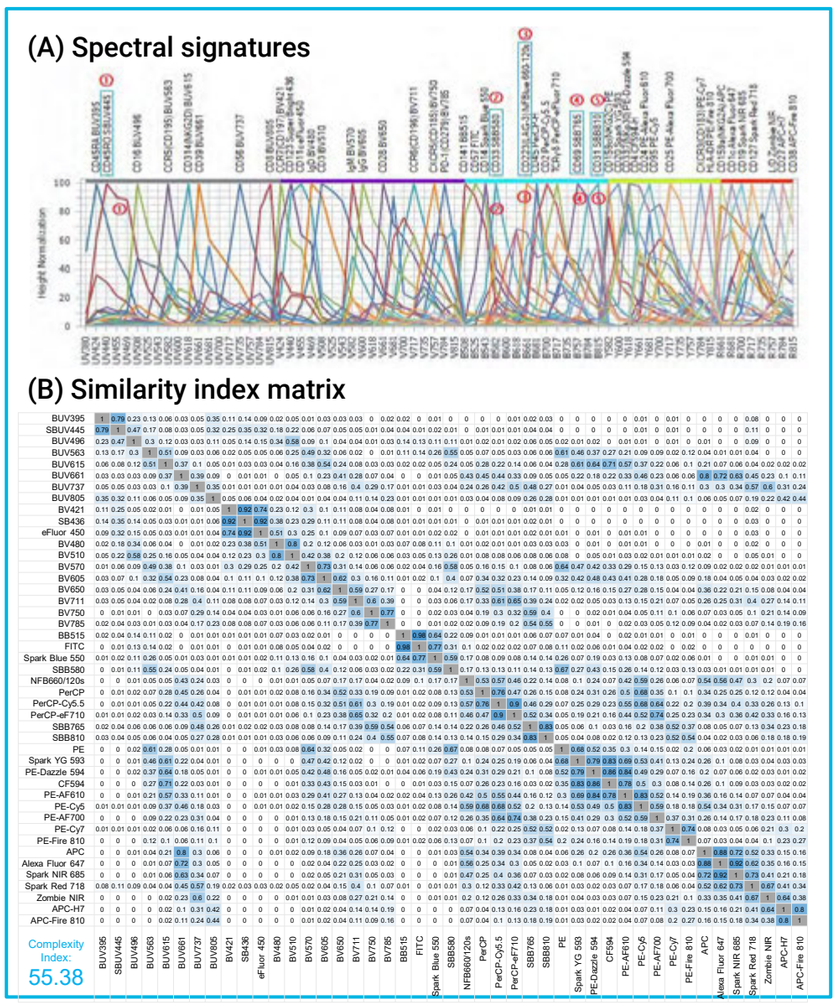



**Figure 2. Spectral signatures and Similarity Index Matrix for the 45-color panel on the NovoCyte Opteon™ flow cytometer. (A)** Spectral signatures. The five added dyes were filled in spectral gaps between the current repertoire of fluorochromes. **(B)** Similarity Index Matrix. The Complexity Index is 55.38, just 1.66 higher than 53.72 according to published data[1].

## Results and Discussions

### Gating strategy

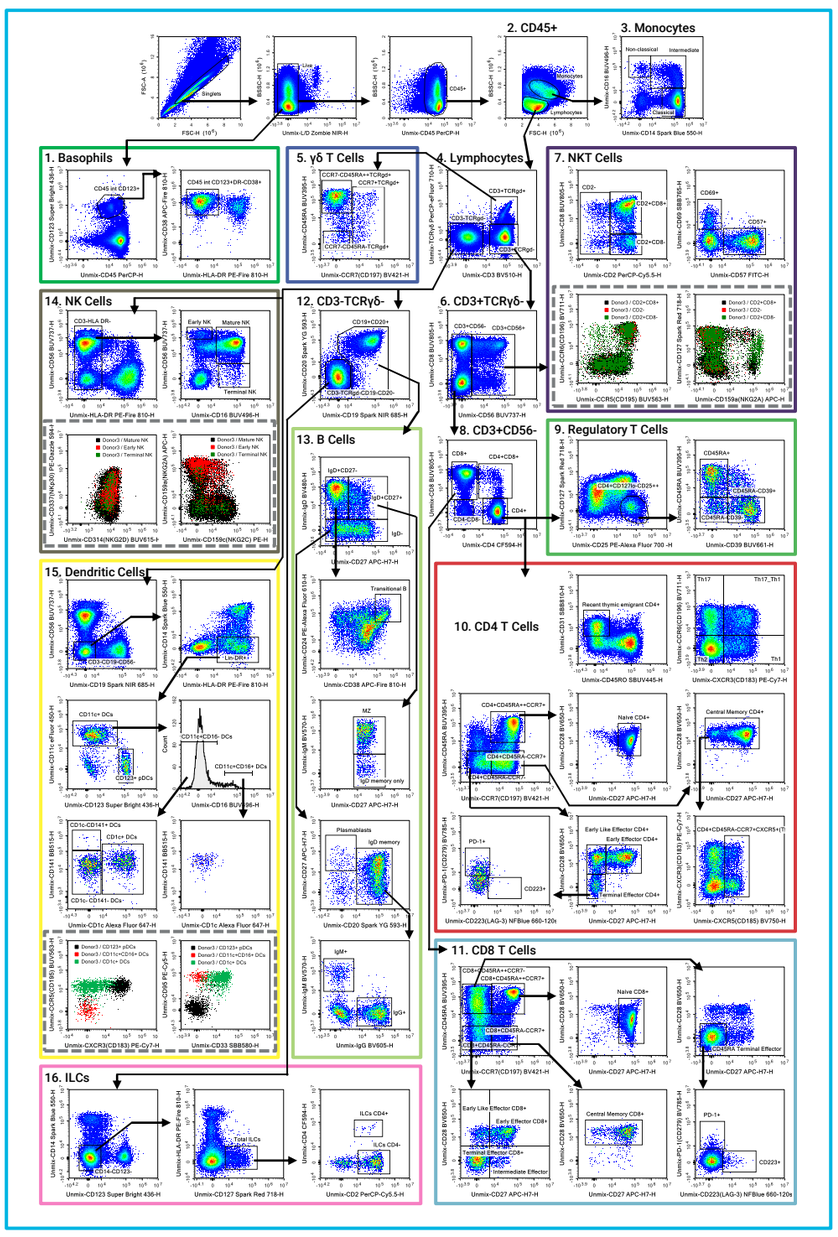
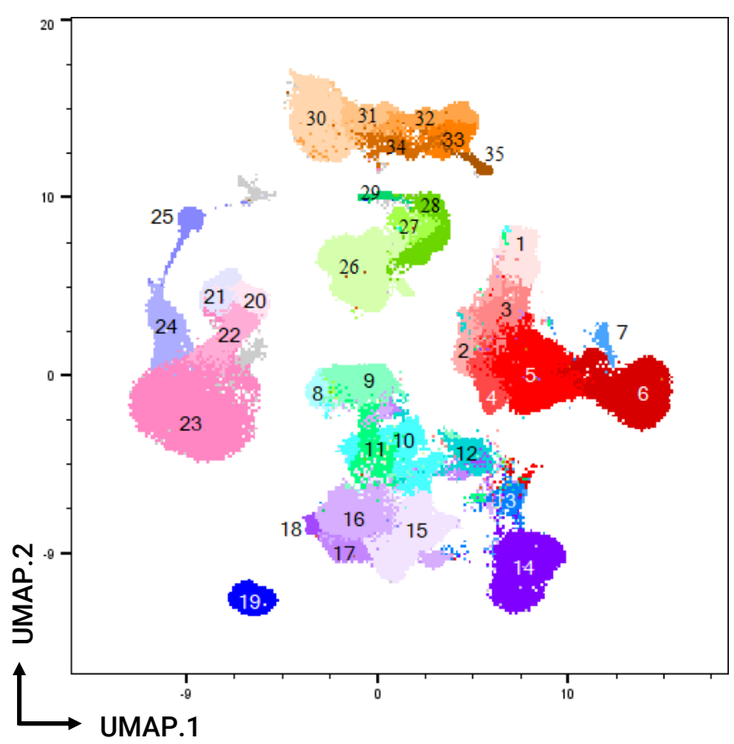



**Figure 3. Gating strategy to identify the main immune cell subsets.** All leukocyte subsets can be clearly distinguished by manual gating, including **(1)** Basophils, **(3)** Monocytes, **(5)** γδT cells **(7)** NKT cells, **(9)** Tregs, **(10)** CD4 T cells , **(11)** CD8 T cells, **(13)** B cells, **(14)** NK cells, **(15)** Dendritic cells, **(16)** lymphoid cells (ILCs). All data presented here are derived from frozen PBMCs of one healthy donor.

## Results and Discussions

### High dimensional analysis

(A) FlowSOM cluster over UMAP




| | | | | | |
|---|---|---|---|---|---|
| 1 | Terminal Effector CD4+ | 13 | CCR7+CD45RA+TCRgd+ | 25 | CD123+ pDCs |
| 2 | Early Like Effector CD4+ | 14 | Naive CD8+ | 26 | Mature NK |
| 3 | Early Effector CD4+ | 15 | Terminal Effector CD8+ | 27 | Terminal NK |
| 4 | CD45RA-Treg | 16 | CD45RA++CCR7- CD8+ | 28 | Early NK |
| 5 | Central Memory CD4+ | 17 | Early Like Effector CD8+ | 29 | ILCs |
| 6 | Naive CD4+ | 18 | Central Memory CD8+ | 30 | IgD+CD27- B cells |
| 7 | CD4-CD8- | 19 | Basophils | 31 | MZ B cells |
| 8 | CD57+CD159a+ NKT | 20 | Non-classical Monocytes | 32 | IgM+ B cells |
| 9 | CCR7-CD45RA-TCRgd+ | 21 | CD1c- DCs | 33 | IgG+ B cells |
| 10 | CD2+CD8+ NKT | 22 | Intermediate Monocytes | 34 | IgD memory only |
| 11 | CCR7-CD45RA++TCRgd+ | 23 | Classical Monocytes | 35 | Plasmablasts |
| 12 | CD2-CD69+ NKT | 24 | CD1c+ DCs | | |

(B) UMAP across donors

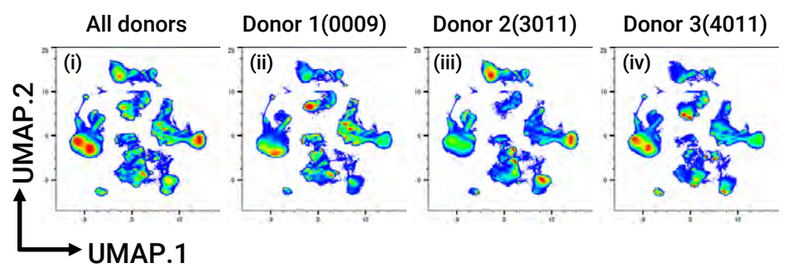



**Figure 4. High dimensional analysis.** After platelets, debris, doublets and dead cells were excluded, the cells in the live CD45+ gate from each donor are reduced to 500,000 events and concatenated into one file, then UMAP and FlowSOM are performed separately based on all markers except CD45 and live/dead dye. **(A)** UMAP with FlowSOM cluster overlay. **(B)** UMAP results across donors: (i) All donors, (ii) Donor 1(0009), (iii) Donor 2(3011), and (iv) Donor 3(4011).

## Conclusions

- We successfully added more dyes to the original OMIP-069 panel to be able to detect more biomarkers.
- All immune subsets could still be accurately and clearly distinguished without affecting the dimensionality reduction and clustering result of the original markers even though more highly overlapping fluorochromes were included in this 45-color panel.
- The results demonstrate the performance and high accuracy of the Agilent NovoCyte Opteon as a spectral flow cytometer.
- Researchers have more choices and can easily generate highly complex flow cytometry panels up to 45 colors in a single tube to address their biological questions.

## References

[1] Park LM, Lannigan J, Jaimes MC. OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood. Cytometry A 2020;97:1044–1051.